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## Murein-metabolizing enzymes from Escherichia Coli

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## SUMMARY

This thesis reports on two murein-metabolizing enzymes from *Escherichia coli*. The two lytic transglycosylases catalyze the cleavage of the  $\beta$ -1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the murein-polymer, yielding muropeptides with a 1,6-anhydro-muramic acid residue.

Studies on bacterial transglycosylases are of interest for at least three reasons. In the first place, such studies contribute to a better understanding of the metabolism of murein. In the second place, these enzymes can be used for the production of pharmacologically active compounds by specific degradation of murein *in vitro*. In the third place, a 3D-structure might lead to the design of selective inhibitors and thus to the development of a new class of antibiotics.

Chapter 1 gives an introduction concerning murein-metabolism, the specific activities of murein hydrolases with emphasis on the lytic transglycosylases, and the biological activities of muropeptides with emphasis on 1,6-anhydro-muropeptides.

In Chapter 2, we describe the nucleotide sequence and overexpression of the *slt* gene encoding the soluble lytic transglycosylase (Slt70) with a molecular mass of 70 kDa. A 250-fold overproduction was tolerated by the bacterial cell and has resulted in a simplified purification procedure for the purification of Slt70 in 100 mg amounts.

Chapter 3 reports on the nucleotide sequence and overexpression of the *mlt* gene which was supposed to encode the second lytic transglycosylase, being membrane-bound (Mlt), with a molecular

mass of 35 kDa. However, a 500-fold overproduction of Mlt did not result in a parallel increase in murein hydrolase activity. This has led to the suggestion that we are most likely not dealing with a gene that encodes a lytic transglycosylase but a cytoplasmic membrane-bound protein with unknown function.

In Chapter 4, we describe the solution of the problem discussed in Chapter 3. A second soluble lytic transglycosylase (Slt35), with a molecular mass of 35 kDa, was purified from an *E. coli* strain which was chromosomally deleted for the *slt* gene encoding Slt70. Polyclonal antibodies, previously being raised against Mlt, cross-react with Slt35 indicating that Slt35 was present in the Mlt-preparations in addition to a cytoplasmic membrane-bound protein of 35 kDa. Therefore, the second transglycosylase in *E. coli* is not a membrane-bound protein as was stated earlier.

Chapter 5 describes the crystallization of Slt70. The crystals diffract in the X-ray beam to 2.8 Å resolution.

The complete 3D-structure of Slt70 has been determined recently (see cover) by Andy-Mark Thunnissen in the group of Bauke Dijkstra at the Department of Chemical Physics at the University of Groningen. Slt70 consists of two domains. The N-terminal 360 amino acids represent a non-globular domain with a horse-shoe-like structure and possesses exclusively  $\alpha$ -helices as secondary structure elements. The C-terminal domain (amino acid 384 to 618) is a globular domain and shows structural similarity to T4-lysozyme which has already resulted in the identification of possible catalytic

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residues. This does not cover the whole domain. This protein, with a molecular mass of 35 kDa, has led to the suggestion that we are most likely not dealing with a gene that encodes a lytic transglycosylase but a cytoplasmic membrane-bound protein with unknown function.

The enormous molecular mass of 35 kDa, the similarity of the amino acid sequence of Slt35 to the amino acid sequence of Slt70, and the structure of Slt35, lacking a domain, suggest that the gene encoding Slt35 might prove this.

The biotechnological application of Slt35 is described in Chapter 6. A quantitative degradation of the murein polymer to the muropeptides, 1,6-anhydro-muropeptides, by digestion of the murein with the transglycosylase Slt35 and two murein enzymes (MepA) is essential for a simple batch process for the large scale production of muropeptides. The hydrolases had a multiple use of 100-fold, achieved by immobilization in a matrix. Both domains

residues. This C-terminal domain does not cover the hole in the N-terminal domain. This enormous hole in the protein, with a diameter of about 25 Å, has led to the speculation that at least one and possibly two sugar chains are passing through the hole. Whether Slt70 degrades *in vivo* one sugar chain or two crosslinked chains, or maybe even two chains connects, will be studied in the near future.

The enormous difference in the molecular masses of Slt70 and Slt35 and the similarity of the N-terminal 26 amino acids of Slt35 with a stretch of amino acids in the middle of the primary structure of Slt70, suggest that Slt35 is lacking a domain similar to the N-terminal domain of Slt70. Cloning of the gene encoding Slt35, its nucleotide sequence, and 3D-structure of Slt35 might prove this.

The biotechnological part of this thesis is described in Chapter 6, 7, and 8. For quantitative degradation of the murein polymer to the monomeric sleep-inducing 1,6-anhydro-muropeptides, a combined digestion of the isolated polymer with a transglycosylase and at least one of the two murein endopeptidases (PBP4 and MepA) is essential. In order to develop a simple batch procedure for the large-scale production of 1,6-anhydro-muropeptides, the purification of the hydrolases had to be simplified and multiple use of the enzymes had to be achieved by immobilizing them on a matrix. Both demands could be fulfilled

by construction and overexpression of the murein hydrolases as Staphylococcal Protein A gene fusions. The N-terminal domain of Protein A consists of five regions possessing high affinity to the F<sub>c</sub> part of IgG.

Chapter 6 reports on the construction of a suitable vector for the controlled high-level overproduction of Staphylococcal Protein A (SpA) fusion proteins in the periplasm of *E. coli*. This vector is used in Chapter 7 for the overproduction of Slt, PBP4, and MepA as SpA fusion proteins. The SpA fusion proteins were stably overproduced and account for 5, 5, and 0.5 mg/liter culture for SpASlt, SpAPBP4, and SpAMepA, respectively.

The stability of the SpA fusion proteins *in vitro* as well as the *in batch* degradation of murein, and purification of the 1,6-anhydro-muropeptides are described in Chapter 8. The SpA fusion proteins, immobilized on IgG Sepharose, are proteolytically sensitive, which resulted in a complete degradation of the SpA portion of the fusion proteins thereby releasing the murein hydrolases in intact and enzymatically active form into the supernatant. Proteolytic degradation could be prevented by PHMB or EDTA. In the presence of PHMB, the immobilized fusion proteins were enzymatically active and could be used for the batch production of biologically active 1,6-anhydro-muropeptides, which successively were separated by HPLC.